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Comparison of New Diagnostic Tools for Management of Pediatric Mediterranean Visceral Leishmaniasis

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New techniques are available for diagnosing leishmaniasis, but their efficacy in the identification of pediatric visceral leishmaniasis (VL) has not been compared with that of traditional methods. Blood, bone marrow, and urine samples were taken from 25 children with VL during their first clinical episode, 22 days after the start of treatment with liposomal amphotericin B (3 mg/kg/day on 6 days over a 10-day period), and when a relapse was suspected during follow-up. The results obtained suggest that antibody detection techniques, the antigen detection in urine (KAtex kit), and *Leishmania* nested PCR (LnPCR) analysis of the blood could be used for diagnosis of the first clinical episode. After treatment, clinical improvement was associated with negativization of Novy-MacNeal-Nicolle culture and microscopy of bone marrow aspirate, KAtex test, and LnPCR blood analysis results. Interestingly, LnPCR analysis of the bone marrow aspirate showed that sterile cure was not achieved in eight patients, two of which suffered a relapse within 10 to 20 weeks. All of the new noninvasive techniques tested showed high diagnostic sensitivity. However, LnPCR analysis of the bone marrow was the most sensitive; this test was able to detect the persistence of parasites and predict potential relapses.

Visceral leishmaniasis (VL) is a parasitic disease caused by two *Leishmania* species: *L. donovani* and *L. infantum*. The parasite is transmitted through the bite of an infected phlebotomine sandfly. *Leishmania* parasites are obligate intracellular protozoa, which by multiplication into phagocytic mononuclear system cells cause a febrile hepatosplenic syndrome with reductions in the erythrocyte, leukocyte, and platelet counts. If diagnosis is not made early and appropriate treatment initiated, this disease can be fatal.

VL is endemic in 62 countries; 200 million people are at risk, and 500,000 new cases occur annually worldwide (14, 15, 39). Mortality data reported by the World Health Organization records 57,000 deaths due to VL in 1999 and 41,000 in 2000 (38, 39), although these figures are mere estimations. In the Mediterranean basin, *L. infantum* is the causative agent of VL and sandflies of the genus *Phlebotomus* are the vector. In this environment the disease is traditionally more prevalent in children than in adults (ratio, 7:3), the median age among the former being less than 4 years (21).

Ideally, all VL cases should be confirmed by direct observation of the parasite in bone marrow or spleen aspirate, either by microscopy or Novy-MacNeal-Nicolle (NNN) culture, but this is a painful, invasive method, and it is sometimes insufficiently sensitive. There is, therefore, much interest in the use of noninvasive biopsies together with more sensitive diagnostic techniques.

Serological tests for the detection of antileishmanial antibodies, including the immunofluorescent-antibody test (IFAT) (4), enzyme-linked immunosorbent assay (ELISA) (19), and the direct agglutination test (18), are well standardized. Recombinant *Leishmania* antigens, such as rK39, have recently been introduced for use in serological tests in both ELISA and immunochromatographic test (dipstick) formats. Reaction to this antigen is thought to be associated with an active episode of VL (5), although antibodies to the rK39 antigen remain present after successful therapy (40). There are, however, several intrinsic problems with serological assays, including cross-reactions with antibodies against other pathogens and the fact that most of these tests cannot readily distinguish between current, subclinical, or past infection.

In principle, an antigen detection test should provide a better means of diagnosing VL, since antigen levels should be broadly correlated with the parasite load. They could also offer an ideal alternative to antibody detection systems in immunocompromised patients, as in *Leishmania*/human immunodeficiency virus (HIV)-coinfected patients who present a lower antibody response (1).

In chronic infections, such as VL, the detection of antigens in serum is complicated by the presence of high levels of antibodies, circulating immune complex, serum amyloid, rheumatoid factors, and autoantibodies, all of which may mask immunologically important antigenic determinants or competitively inhibit the binding of antibodies to free antigen (35). Many of these problems may be avoided by searching for antigens in the urine. Several studies have demonstrated *Leishmania* antigens in the urine of VL patients and animals using different techniques including double countercurrent immunoelectrophoresis (22), ELISA (3, 20, 36), and Western blotting (13). Recently, a latex agglutination test (KAtex kit) to detect *Leishmania* antigen in urine showed 100% specificity and 47 to 100% sensitivity in different studies with immunocompetent

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patients (2, 32) and 96 to 100% specificity and 85 to 100% sensitivity in immunodepressed patients (31, 37).

On the other hand, different PCR assays have been developed for diagnosing leishmaniasis. This technique has been shown to be more useful than conventional diagnostic methods, since its sensitivity is greater (17, 29). Several studies have shown that a positive PCR result is related to the presence of living parasites (23, 28), while a negative result is obtained after parasitological cure (8, 16, 27).

In this work *Leishmania* nested PCR (LnPCR) was used for the diagnosis and the monitoring of treatment of pediatric VL. This technique has a high diagnostic sensitivity with respect to the first clinical episode of VL in *Leishmania*/HIV-coinfected patients (95% sensitivity when performed on peripheral blood and 100% on bone marrow samples [12]).

Currently there is a certain momentum with respect to the search for new diagnostic techniques for VL, but those developed have mostly been validated for adults. The present study compares the efficacy of molecular (LnPCR), antibody (IFAT, soluble *Leishmania* antigen ELISA [SLA-ELISA], rK39 ELISA, rK39 dipstick), and antigen detection techniques (KAtex kit) with one another and with traditional bone marrow aspirate NNN culture and microscopy in the diagnosis of clinical VL and follow-up monitoring with immunocompetent children.

MATERIALS AND METHODS

Patients. The study subjects were 25 pediatric patients enrolled in a prospective, open, multicenter clinical trial; all were studied during their first clinical episode of VL and during follow-up. Diagnosis of VL was made on the basis of clinical signs and symptoms and the results obtained with the different diagnostic techniques tested (IFAT, SLA-ELISA, rK39 ELISA, rK39 dipstick, KAtex kit, bone marrow NNN culture and microscopy, and LnPCR analysis of the blood and bone marrow).

After diagnosis, the patients were treated with liposomal amphotericin B (AmBisome; Gilead Sciences, Inc.) at a dose of 3 mg/kg of body weight/day on 6 days over a 10-day period (on days 1, 2, 3, 4, 5, and 10), reaching a total dose of 18 mg/kg.

The mean age of the patients was 2 years (range, 5 months to 10 years, although 24 out of 25 patients were under 4 years old). One-half of the patients were male and half female. No child was HIV positive.

Signed, informed consent to be included in this study was provided by the parents or guardians. The study was approved by the ethics committee of each participating hospital.

Sample collection. Peripheral blood, bone marrow aspirate, and urine samples were taken for diagnostic purposes during the first clinical episode of VL and again at 22 days after the start of treatment. When a relapse episode was suspected, samples were taken during the active phase.

At each sampling point, 5 ml of peripheral blood was collected in a tube containing an anticoagulant (EDTA or heparin). In addition, 200 μl of bone marrow aspirate was collected and diluted 1:1 in NET10 buffer (NaCl, 10 mM; EDTA, 10 mM; Tris HCl, 10 mM, pH 8.0). Between 5 and 10 ml of urine was also collected. Serum was obtained by centrifugation of the blood samples at 2,000 × *g* for 15 min.

IFAT. IFAT analysis was performed by following a standard method (6). The antigen was prepared from promastigotes of the *L. infantum* zymodeme MON-1 international reference strain MHOM/FR/78/LEM-75. Antibody binding was detected using fluorescein isothiocyanate-conjugated sheep antihuman immunoglobulin G (heavy and light chains). The threshold titer for positivity was 1/80. This cutoff value was established by combining the highest sensitivity and specificity obtained using pooled serum from VL patients from endemic areas and comparing to the results obtained with pooled serum from healthy subjects.

SLA-ELISA. A promastigote lysate served as the antigen source for ELISA analysis of the serum samples. Microtiter plates were coated with soluble *L. infantum* antigen (1 μg/well) prepared as described by Scott et al. (33). Each serum sample was tested in duplicate (at a dilution of 1/100); positive and negative controls were included on each plate. Bound antibody was detected using goat antihuman immunoglobulin G–biotin and streptavidin–horseradish peroxidase conjugates. The optimum dilutions for the test sera and conjugates were determined by chessboard titration. An optical density (OD) of 0.249 at 405 nm was used as the cutoff for seropositivity; this was the mean OD for the sera of healthy subjects from VL-endemic areas plus three standard deviations.

rK39 ELISA. Antibody levels against the rK39 antigen were tested as previously described (7). The working dilutions of the antigen and the serum were determined by chessboard titration. The ideal antigen/serum combination giving the best differentiation between negative and positive sera was chosen (50 ng/well; 1:100 serum dilution). Each serum was assayed in duplicate. Bound antibody was detected with protein-A-conjugated horseradish peroxidase. The OD of the well was read at 405 nm; the cutoff value was 0.082, i.e., the mean OD of negative controls from VL-endemic areas plus three standard deviations.

rK39 immunochromatographic test. The dipstick format used was the Kalazar Detect Rapid test (InBIOS International, Seattle, WA). Antibody detection was performed both with serum samples (according to the manufacturer's instructions) and blood (after mixing 50 μl of peripheral blood and 4 drops of the Chase Buffer solution provided with the kit).

Latex agglutination test. Urinary antigen detection was performed with the KAtex kit (Kalon Biological Ltd., Aldershot, United Kingdom). Two-hundred-fifty-microliter urine samples were mixed with antibody-sensitized latex particles. Agglutination was determined visually after stirring for 2 min.

NNN culture. One hundred microliters of bone marrow aspirate dilution was cultured in NNN medium at 27°C and examined by light microscopy every week for promastigote forms before subculturing with fresh medium. Subcultures were performed for 4 weeks before a negative result was returned.

Microscopy. Bone marrow aspirate smears were stained with Giemsa stain. Two experienced microscopists independently examined each slide for at least 1 h looking for amastigotes. These were confirmed by examination at a magnification of ×1,000.

DNA extraction. One hundred microliters of peripheral blood and 100 μl of bone marrow aspirate dilution were used as DNA sources. Three hundred microliters of NET10 buffer and 40 μl of 10% sodium dodecyl sulfate were added to each sample. After incubation at 70°C for 1 h, the samples were subjected to classical phenol-chloroform DNA extraction and ethanol precipitation. The DNA obtained was diluted in 100 μl distilled sterile water and stored at 4°C until use.

Diagnostic PCR. LnPCR was performed to detect leishmanial DNA in blood and bone marrow using 10 μl of the extracted DNA according to the method of Cruz et al. (12) with modifications that improved performance. In the first reaction, 15 pmol of primers R221 and R332 were used; primers U and H330R were avoided. For the second reaction, 10 μl of a 1/40 dilution of the first PCR product was used as a template in the presence of 7.5 pmol of primer R223 and 3.75 pmol of primer R333. The quantity of DNA tested by PCR was the equivalent to 10 μl of peripheral blood or 5 μl of bone marrow aspirate.

Differentiation between relapses and reinfections. When a relapse episode was suspected, kinetoplast DNA (kDNA) seminested PCR (snPCR)–restriction fragment length polymorphism (RFLP) was performed on bone marrow samples to discriminate between a relapse and reinfection by a new *Leishmania* strain (26). Briefly, the kDNA amplified by snPCR was digested with restriction enzymes and subjected to electrophoresis to obtain specific band patterns for each strain. The RFLP analysis of the strains obtained before and after treatment established whether the new episode was caused by the same parasite responsible for the initial infection.

Small changes were made to the kDNA snPCR protocol to ensure better performance. The first reaction was performed in a 50-μl final volume with 10 μl of template, 15 pmol of DRJ and KLK2 primers, and 1.4 U of *Tth* DNA polymerase. The annealing temperature was raised to 70°C and the number of cycles reduced to 30. In the second reaction, 10 μl of a 1/40 dilution of the first PCR product was used as a template.

Restriction analysis of the purified second PCR products (using the GFX PCR DNA and Gel Band Purification kit; Amersham Biosciences, Little Chalfont, United Kingdom) was performed with RsaI restriction enzyme according to the manufacturer's instructions (Roche Diagnostics GmbH, Penzberg, Germany). RFLP analysis was performed by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) following the manufacturer's instructions.

Statistical analysis. Sensitivity values recorded by each diagnostic method were tested for significance by the chi-square test.

TABLE 1. Signs and symptoms during the first clinical episode of VL

Symptom	No.[a] (%) of patients
Fever	25/25 (100)
Splenomegaly	24/25 (96)
Hepatomegaly	18/25 (72)
Anemia	11/25 (44)
Thrombocytopenia	6/25 (24)
Neutropenia	2/25 (8)
Leukopenia	2/25 (8)
Pancytopenia	4/25 (16)
Anorexia	2/25 (8)
Diarrhea	1/25 (4)
Abdominal pain	1/25 (4)
Paleness	1/25 (4)
Meningeal syndrome	1/25 (4)
Hypoproteinemia	1/25 (4)

[a] No. of patients with symptom/no. studied.

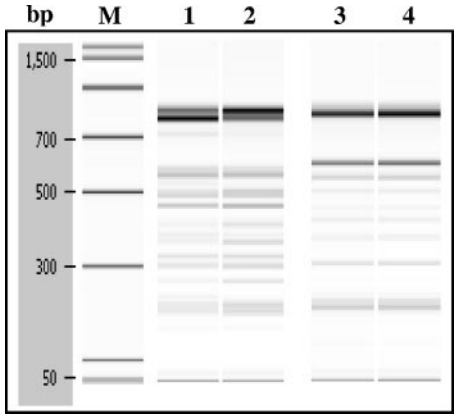

FIG. 1. RFLP patterns for patients P-21 and P-24 during the first (lanes 1 and 3, respectively) and second (lane 2 and 4, respectively) episodes. M, molecular weight marker.

RESULTS

Diagnosis of the first clinical episode. Fever, splenomegaly, and hepatomegaly were detected in almost all patients during the first clinical episode of VL. Other clinical manifestations seen less frequently were anemia, thrombocytopenia, neutropenia, leukopenia, pancytopenia (some kind of cytopenia was reported in 60% of the patients), anorexia, diarrhea, abdominal pain, paleness, meningeal syndrome, and hypoproteinemia. Table 1 shows the different clinical signs and symptoms noted and their frequencies.

The sensitivity of the different anti-*Leishmania* antibody detection methods ranged from 89 to 96% (Table 2), although the differences observed were not statistically significant ($P > 0.05$). Antigen detection in urine was performed for 23 patients; 16 gave positive results (69%). Parasites were isolated by bone marrow NNN culture in 11 of the 25 patients (44%), and amastigote forms were observed in 16 out of 24 bone marrow smears analyzed (67%). *Leishmania* DNA was detected in peripheral blood in 19 out of 24 patients (79%) and in bone marrow in 24 out of 24 patients studied (100%).

Posttreatment control. Posttreatment monitoring was performed with 20 of the 25 patients (80%) for an average of 22 days after the start of treatment. At this stage all patients were asymptomatic and no clinical signs related to VL were recorded.

The presence of anti-*Leishmania* antibodies was detected in all patients by all of the techniques employed, with the exception of SLA-ELISA and rK39 ELISA, which returned a negative result for one patient (P-24).

The KAtex analysis, bone marrow NNN culture, and microscopy returned negative results for all patients during posttreatment control. LnPCR analysis of peripheral blood returned a positive result only for patient P-24; LnPCR analysis of the bone marrow was positive for 8 out of 19 patients, indicating persistence of infection despite clinical recovery.

Follow-up. A relapse episode was suspected for patients P-21 and P-24; both returned a positive posttreatment bone marrow LnPCR analysis result; patient P-24 also returned a positive peripheral blood LnPCR analysis result. Another six patients who maintained a posttreatment positive LnPCR bone marrow analysis result did not relapse and remained asymptomatic during the study period.

Although the symptoms of patient P-21 were mild, clinical relapse was strongly suspected, and samples were taken on day 49 after that taken on day 22 following the start of treatment. Results of all antibody detection techniques and LnPCR bone marrow analyses were positive, while antigen detection in the urine, bone marrow microscopy and culture, and LnPCR analysis of the peripheral blood were negative. For patient P-24, relapse was suspected on day 113 after the last sample was taken (day 22 after the start of treatment); the symptoms were fever, leukopenia, and thrombopenia. All diagnostic techniques returned a positive result for this second episode.

The results of kDNA snPCR-RFLP performed on the initial and second episode samples were compared to distinguish between a relapse and newly acquired infection. The coincidence of the RFLP patterns indicated a relapse for both patients (Fig. 1).

TABLE 2. Detection of anti-*Leishmania* antibodies by different methods during first clinical episode of VL

Anti-*Leishmania* antibody detection method	No.[a] (%) of positive patients[b]
IFAT	22/23 (96)
SLA-ELISA	17/18 (94)
rk39 ELISA	16/18 (89)
Serum rK39 dipstick	23/24 (96)
Blood rK39 dipstick	23/25 (92)

[a] No. of positive patients/no. tested.
[b] Note that some of the patients were not analyzed by all the diagnostic techniques.

DISCUSSION

The mean age, sex ratio, and clinical features of the present patients are similar to those described in other studies on pediatric Mediterranean VL (9, 25).

For the diagnosis of the first clinical episode, the combination of any antibody detection technique plus KAtex and LnPCR analysis of the peripheral blood yielded better results than the traditional invasive diagnostic procedure (NNN culture or mi-

croscopic analysis of bone marrow aspirate; used to demonstrate the presence of the parasite). Statistically significant differences at the level of P values of <0.05 were observed when serological techniques were compared to NNN culture or microscopy, with the exception of rK39 ELISA versus microscopy ($P = 0.0943$). These noninvasive sampling methods allow antibodies, antigens, and parasitic DNA to be detected and permit an accurate diagnosis to be made. One of the aims of this work was to evaluate the diagnostic usefulness of the KAtex kit for pediatric VL. The 69% sensitivity recorded for this technique with the present children was in agreement with the range of 47 to 100% reported by other authors (2, 32) and virtually the same as that of microscopy (67%), as reported in *Leishmania*/HIV-coinfected patients (37). No statistically significant differences in sensitivity were observed when the KAtex kit was compared to bone marrow aspirate microscopy and NNN culture or blood LnPCR analysis. And the serological techniques and bone marrow aspirate LnPCR analysis shown higher sensitivity than this method ($P < 0.05$). Although its sensitivity is acceptable, future work to improve the sensitivity of this test could be very interesting due to its high specificity and easy handling.

Clinical cure was confirmed for all patients at posttreatment control. This was defined as a lack of clinical manifestations of disease, the absence of urinary leishmanial antigens (supporting the idea that the KAtex kit is good for the detection of active disease only), and the inability to isolate parasites by NNN culture or detect them by microscopy. The results of the LnPCR analysis of the blood also appear to provide a good marker of cure, since they remained positive only for one patient (P-24), who later relapsed. Antibody detection remained positive after treatment, as expected, and no variation in antibody titer was observed either with IFAT, SLA-ELISA, or rK39 ELISA analysis.

A positive LnPCR bone marrow analysis after treatment was related with a subsequent relapse in two out of eight cases. The permanence of infection after clinical cure for the other six patients is not unusual; asymptomatic infection has been described elsewhere (24, 34). Six patients suffered no new episode during the follow-up period (more than a year). When relapse was associated with only mild clinical features (patient P-21), *Leishmania* infection was detectable only by LnPCR analysis of the bone marrow. For patient P-24, in whom patent clinical signs were seen, all diagnostic techniques were positive.

Pizzuto et al. (30) considered VL highly probable even in patients without parasitological evidence but with suggestive clinical signs and symptoms and positive IFAT titers. In Bangladesh, Chowdhury et al. (10, 11) reported VL in patients with positive serology but with no parasitological evidence of infection. In such situations, and because of the serious therapeutic implications of an incorrect or late diagnosis of VL, there is a pressing need for an accurate laboratory test that can confirm the clinical diagnosis. This is made manifest in the present work, in which roughly 30% of patients could not be diagnosed by demonstration of the parasite. Only the LnPCR analysis of the bone marrow, the most sensitive technique (100%; $P < 0.05$ compared to bone marrow aspirate NNN culture and microscopy), was capable of detecting parasites in all cases.

The KAtex and LnPCR blood techniques are reliable in comparison with microscopy and NNN culture of bone marrow aspirate. These noninvasive procedures may therefore be used as an aid in the diagnosis of VL in pediatric patients. Nevertheless, higher sensitivity is obtained with LnPCR analysis of the bone marrow, an invasive procedure. Taking into account the high sensitivity of the serological techniques, a good option would be to combine them with KAtex and LnPCR blood analysis as an initial diagnostic step; if these prove negative when clinical suspicion persists, an LnPCR analysis of the bone marrow should be performed. LnPCR analysis of the bone marrow is proposed as a definitive diagnosis method and as a test of sterile cure (i.e., to rule out cryptic infections that could cause a relapse). LnPCR analysis of peripheral blood has been shown to be more sensitive than NNN culture (79% *versus* 44%; $P < 0.05$) and microscopy (79% *versus* 67%; $P = 0.450$) of bone marrow aspirate; however, further work is needed to increase the sensitivity of this analysis in order for invasive tests to be used less often. A possible approach could be the use of peripheral blood mononuclear cell concentrate instead of whole blood.

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